CARBON-14 FIXATION IN THE POLLEN OF YELLOW LUPINE (*LUPINUS LUTEUS* LINN.)

by

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INTRODUCTION

Carbon assimilation studies have usually been associated with the unicellular algae or with the chlorophyll-containing vegetative organs of green plants. It has been reported, however, that certain species of plants also contain chlorophyll in their pollen grains (16). This is not unexpected as pollen grains are considered to be degenerate gametophytes which at one stage in their ontological development carried on the life processes which are inherent in green gametophytes of today. Since chlorophyll is closely associated with isotopic tracer studies of photosynthesis, an interesting problem presented itself which could be conducted with chlorophyll-containing pollen grains. Consequently, the purpose of this investigation was to study carbon-14 fixation in chlorophyll-containing pollen grains to determine if the chlorophyll is functional photosynthetically.

SURVEY OF LITERATURE

Likely, the discovery of chlorophyll in pollen grains was initiated through studies of plastid morphogenesis (1, 9). Two German workers, Ruhland and Wetzel (16), reported chlorophyll in pollen while studying plastid origination. In 1924, they published findings which indicated the presence of chloroplasts in the generative cells of yellow lupine (Lupinus luteus Linn.), narcissus (Narcissus incomparabilis Mill.), and crocus (Crocus vernus All.) of some 50 species sampled. They based their conclusions on two methods of detection, luminescent microscopy and microchemical reactions.

When Ruhland and Wetzel observed germinating pollen microscopically under ultraviolet light, a diffuse but distinct red fluorescence was observed throughout the spindle-shaped generative cell of the pollen tube in the above named species. Their second method of chlorophyll detection was based on the reduction of silver by chlorophyll granules (11). In the generative cell of those species which gave the fluorescence reaction by one test, silver reduction was observed in the second test. Plate I illustrates their representation of a chlorophyll-containing generative cell which has been impregnated with silver nitrate. They also stated that the chloroplasts gradually lost their chlorophyll as the pollen tubes grew longer and the plastids changed from a circular shape into irregular chondriosome-like structures. Furthermore, that the pollen of yellow lupine contained the most chlorophyll of the three species reported.

Although chlorophyll was known to be intimately related to photosynthetic carbon reduction before these observations of Ruhland and Wetzel, the problem of separating and identifying the intermediary compounds in the photosynthetic process proved to be a formidable barrier as long as only classical methods of analyses were used. In 1939, however, Ruben, Hassid, and Kamen (13) reported a technique for following the mechanism of carbon assimilation through the use of the radioactive isotope carbon-11. They found that radiocarbon was incorporated into carbohydrate not only in the light, but in the dark as well.

The early tracer carbon work was hindered by two factors: One, that the half life of C^{11} is only 22 minutes which made lengthy fractionation procedures impossible. Two, was the lack of analytical procedures for separating the diverse mixture of labeled metabolites

EXPLANATION OF PLATE I

Pollen grain of yellow lupine (*Lupinus luteus* Linn.) impregnated with a 1.0 per cent silver nitrate solution. (After Ruhland and Wetzel (16))

A. Exine

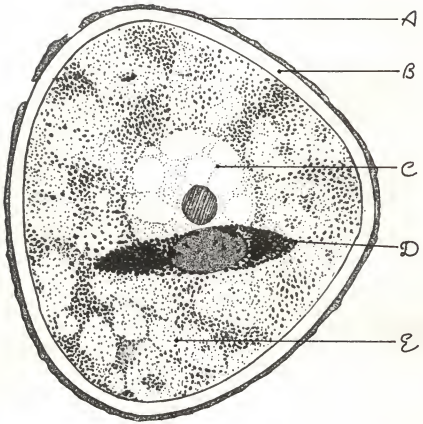
B. Intine

C. Tube nucleus

D. Generative cell

E. Cytoplasm

PLATE I



formed after only a few seconds of photosynthesis. In 1940, it was discovered that the long lived carbon isotope, carbon-14, could be made by a (n,p) reaction from N^{14} (14, 15) and, hence, was available in quantity for such biological research.

By 1948, Calvin and Benson (8), using extraction, precipitation, and ion exchange procedures to separate $C^{14}O_2$ fixation products, found phosphoglyceric acid, malic acid, and alanine to be significantly labeled after 30 seconds of photosynthesis with $C^{14}O_2$. Nevertheless, the analytical procedures used by groups working with carbon-14 were involved, time consuming, and often unreliable. With the development of paper chromatographic methods, however, investigators began to apply the combination of tracer studies and paper chromatography-autoradiography to the separation and identification of photosynthetic products. By 1950, Benson and co-workers (4) had identified phosphoglyceric acid, phosphopyruvic acid, malic acid, and sometimes glyceric acid as products of carbon assimilation during short photosynthetic periods. As longer exposures to $C^{14}O_2$ were made, radioactivity was found not only in the above compounds, but also in aspartic acid, alanine, serine, glycine, glycollic acid, triose phosphates, hexose phosphates, hexose diphosphates, and sucrose.

In early work on dark-fixation using labeled carbon dioxide, the possibility of respiratory carbon fixation was not fully comprehended. It is now generally accepted that there are two types of dark fixation, a "photosynthetic" and a "respiratory" (12). The former type is obtained when plants are exposed to $C^{14}O_2$ immediately following a period of illumination in the absence of carbon dioxide. In this case the labeled products are almost the same as with short exposures

(approximately 15 seconds) in the light.

The latter type of dark fixation is obtained when the exposure to radioactive $C^{14}O_2$ in the dark does not follow for several minutes after a period of illumination. A much slower rate of fixation of the radiocarbon is observed. This type of fixation, however, also occurs in light. Because the fixation process does not require light energy, it is commonly called "dark," "respiratory," or even "respiratory dark fixation." To remove the connotation of the word "dark," this ubiquitous fixation is called "respiratory fixation" in this study. The labeled products here are malic, succinic, fumaric, citric, glutamic, and aspartic acids, and alanine. These compounds are believed to be labeled by the fixation of carbon dioxide through the reversible carboxylations of the Krebs cycle intermediates. Phosphoenolpyruvate, however, is also labeled but is not a Krebs cycle intermediate. The initial fixation product(s) of respiratory fixation has remained rather elusive, however. In 1935, Wood and Werkman (21) hypothesized that carbon dioxide combined with pyruvate to form oxaloacetate. This reaction has not been experimentally verified, but recent work by Saltman et al. (17) supports this hypothesis. They identified the products of a 6-second dark fixation of $C^{14}O_2$ by excised leaves of Bryophyllum calycinum as malate and aspartate. They suggested that the reaction mechanism involves oxaloacetate as the common, but transient, precursor. Evidence for the occurrence of oxaloacetate in succulent leaves as well as the presence of the enzyme mediating the synthesis of this compound from PEP (phosphoenolpyruvic acid) and CO_2 supports their postulation (19). From their 6-second $C^{14}O_2$ fixation studies they have suggested the following scheme for the initial reactions of respiratory carbon dioxide fixation

by succulent leaves:

$$\text{Carbohydrates} \xleftarrow{\text{glycolysis}} \text{PEP}$$

$$\text{PEP} \xrightarrow[\text{PC}]{+\,CO_2} \text{oxaloacetate}$$

$$\text{Oxaloacetate} \begin{cases} \xrightarrow[\text{MD}]{} \text{malate} \\ \xrightarrow[\text{T}]{+NH_3} \text{aspartate} \end{cases}$$

PC = PEP carboxylase MD = Malic dehydrogenase

T = Transaminase

Although an abundant literature exists on pollen anatomy, morphology, and physiology, no studies could be found on the nature and function of chloroplasts in pollen grains. The work of Ruhland and Wetzel invariably proved to be the ultimate source whenever reference was made to this situation. Similarly, no references were found on carbon assimilation studies in chlorophyll-containing pollen grains.

MATERIALS AND METHODS

Plants and Pollen

Seeds of sweet yellow lupine (Lupinus luteus Linn.) var. Weiko 3 were obtained from the Florida Agricultural Experiment Station at Gainesville, Florida. They were inoculated and planted five per 8-inch clay pot at a depth of one inch in good loam soil. Blossoms were readily obtained on greenhouse-grown plants under a long (16 hour) day. The yellow lupine is an upright annual with a coarse stem and medium

sized digitate leaves. The yellow blossoms have the characteristic leguminous flower, about 1.5 to 2.0 cm in length.

The pollen grains are triangular in shape with minutely pitted walls and are about 158 microns in diameter. Upon magnification they appear as hyaline structures, yellow-white in color. The average blossom contained approximately 0.3 mg of pollen.

Nutrient Medium

Before tracer studies were made a nutrient medium was formulated which would induce pollen tube germination and growth. It was postulated that the development of a pollen tube would constitute a more favorable environment for study by providing a more active metabolic situation. While a complete nutrient solution, including micronutrients, was devised which was quite satisfactory, it was soon found that one consisting of 15 per cent sucrose plus 4 ppm boron sufficed without undue rupturing of the pollen tubes. This was the nutrient medium used in this study. In such a medium, 90 per cent of lupine pollen germinated within 20 minutes after inoculation.

Radiocarbon Used

The radiocarbon used was C^{14} and was acquired in two different chemical forms. One form was obtained from the Nuclear-Chicago Corporation of Des Plaines, Illinois, as a neutral solution of $Na_2C^{14}O_3$ with a specific activity of 1 to 5 mc/mM. Two such solutions with different total activities were used. One had a total activity of 100 μc and was

diluted to 11 μc/100 μl while the other had a total activity of 500 μc and was diluted to 20 μc/100 μl. The other form used was solid $BaC^{14}O_3$ with a specific activity of 29 mc/mM and was obtained from Oak Ridge National Laboratory. An amount of $BaC^{14}O_3$ calculated to release 500 μc was used per exposure.

Method of Exposure to $C^{14}O_2$

The method of exposure to $C^{14}O_2$ consisted of using a 40 ml Stender dish as the exposure chamber. The pollen was dusted onto a concave, etched, glass planchet (2.5 cm in diameter) and then moistened with nutrient medium. A 5 ml beaker was used to hold the radioactive source. Both planchet and beaker were then placed on moistened filter paper which covered the base of the Stender dish. The ground glass lip of the Stender dish was heavily coated with stopcock grease and pressed into the channeled ring of the base. The grease caused the Stender dish to become an air-tight chamber and the moist filter paper maintained a high humidity. Labeled $C^{14}O_2$ was released in the chamber by addition of 0.25 ml of 85 per cent lactic acid introduced into the small beaker containing the carbon-14 source by means of a syringe-septum arrangement. Plate II illustrates the Stender dish, beaker, and planchet used for exposing pollen to $C^{14}O_2$. Three levels of radioactivity were used during the course of this study. The 11 μc and 20 μc levels were obtained by acidification of the $Na_2C^{14}O_3$ solutions of different total activities. Acidification of 3.5 mg of $BaC^{14}O_3$ was used to provide the 500 μc level.

After an exposure the pollen could be easily and rapidly transferred

EXPLANATION OF PLATE II

Apparatus used for making exposures to $C^{14}O_2$

A. Septum

B. Five milliliter beaker

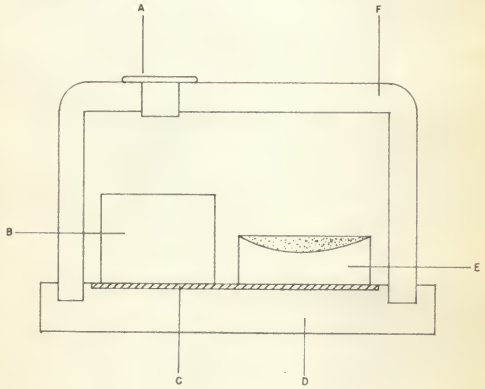
C. Moistened filter paper

D. Stender dish base

E. Concave, etched glass planchet

F. Stender dish cover

PLATE II



from the exposure chamber by carefully dropping the planchet into an extraction beaker.

Exposures were later made in an atmosphere of nitrogen. Here the Stender dish was modified by the addition of inlet and outlet tubes so that the chamber could be flushed with N_2 gas. After the pollen and labeled sodium carbonate were in their respective receptacles and positioned on the Stender dish base, the dish and base were sealed and the chamber flushed with N_2 gas. By sealing off the inlet and outlet tubes simultaneously, a N_2 atmosphere was obtained.

Conditions of Exposures

The same general pattern was followed for all exposures. After releasing the labeled $C^{14}O_2$ from the acidified carbon-14 source, the pollen was allowed to assimilate the labeled carbon dioxide either in light or in darkness for a comparable interval. Effort was made to maintain the total concentration of CO_2 (both labeled and unlabeled) in the exposure vessel to not more than 1.5 per cent. Exposures in light were made in a greenhouse in direct sunlight when the light intensity there was 6,000 foot-candles or higher. This condition was usually obtained between the hours of 11:00 A.M. and 1:00 P.M. Light intensities were measured by a Weston illumination meter, model 603.

Prior to all studies conducted in darkness an effort was made to deplete the pollen cells of any photosynthetic metabolites by maintaining them in complete darkness for at least one hour. In these dark exposures manipulations such as placing of pollen in the exposure chamber, release of $C^{14}O_2$, and transfer of pollen to the extraction beaker

were accomplished under light intensities of 5 ft-c or less of a 50 watt incandescent lamp eight feet or more distant.

During the carbon-14 fixation in the dark the temperature could be maintained at 25°C both inside and outside the exposure without difficulty. During the light exposures, however, direct radiation coupled with the heat trapping effect within the glass exposure chamber required correction. This was accomplished by placing the exposure chamber in a wire mesh basket (10 cm x 10 cm x 5 cm) which had previously been depressed into a container of ice. A thermometer inserted into a similarly exposed chamber served as a control. A temperature of 25° to 28°C could be maintained even when the greenhouse temperature reached 40°C.

Length of Exposure

Varying lengths of exposures to $C^{14}O_2$ were used. They were determined by the level of radioactivity used.

At the 11 μc activity level, the pollen was exposed to the labeled $C^{14}O_2$ for 90 minutes. This comparatively long exposure was used to assure maximum carbon-14 fixation in view of the fact that not all of the pollen grains germinate at the same time and, furthermore, because respiration rates of the pollen grains are not known.

When the activity was increased to 20 μc the exposure was shortened to 45 minutes. It was contended that with the increase in activity, this interval would allow sufficient carbon-14 to be fixed either in the initial products of photosynthetic or in respiratory carbon dioxide assimilation but with minimum continuance of carbon-14 exchange on to secondary metabolites. Later in the study it was decided that exposure

to $C^{14}O_2$ of very high specific activity for a comparatively short interval (1.5 minutes) may produce more easily identified initial products of carbon fixation. Consequently, a light and a dark exposure was made using 3.5 mg of solid $BaC^{14}O_3$ per exposure instead of the $Na_2C^{14}O_3$ solution. Upon acidification with lactic acid, approximately 500 µc of $C^{14}O_2$ were obtained. Twenty-five milligrams of pollen per exposure were allowed to germinate for 25 minutes on the nutrient-moistened planchet before being exposed to $C^{14}O_2$ to assure a high percentage of germinated pollen. At the end of this germinating period the exposure chamber was sealed and the pollen exposed to the labeled carbon dioxide.

Extraction and Lyophilization

After the prescribed exposure, the chamber was opened under a hood or large exhaust fan, thus, sweeping the remaining $C^{14}O_2$ into the atmosphere. The etched glass planchet containing the exposed pollen was immediately dropped into a 50 ml beaker containing about 20 ml of boiling 80 per cent ethanol to stop all enzymatic reactions. The pollen was extracted in the boiling alcohol for 10 minutes and then placed in a micro-homogenizer. After extraction and homogenization, the homogenate was placed in a lyophilizer and reduced in volume to 0.5 ml by the removal of alcohol through freeze-drying at reduced pressure. Plate III illustrates the lyophilizing apparatus used.

Detection and Identification of Radioactivity

For total activity counts 50 µl aliquots were taken from the

EXPLANATION OF PLATE III

Lyophilization apparatus used for reducing the volume of pollen extracts

A. Liquid air cooling finger

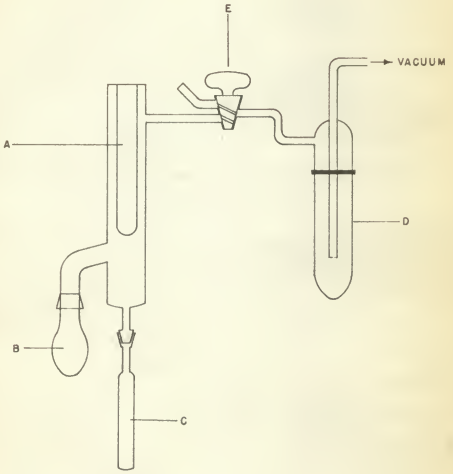
B. Extract volume reduction tube

C. Collection tube for volatile waste

D. Cold trap (immersed in liquid air)

E. Two way stopcock

PLATE III



concentrated extract and added to 10 ml of a scintillating solution. The scintillator contained 1.75 gm of PPO[1], 12.5 mg of POPOP, and 25.0 gm of naphthalene and was diluted to 250 ml with dioxane. The sample was then counted in a Packard model 314-DC Tri-Carb liquid scintillation spectrometer for total fixed activity.

For one dimensional chromatography 100 µl aliquots of the concentrated extract were transferred to Whatman No. 1 chromatography paper (23 cm x 25 cm) and formed into cylinders. These chromatograms were developed one dimensionally in a n-butanol: acetic acid: water (4:1:5 v/v/v) solution by ascending chromatography. The chromatography cabinet consisted of a large bell jar enclosing a glass dish containing the developing solvents. The chromatograms were air dried and redeveloped four times to allow better separation of the sugar moities. After development, the chromatograms were cut into strips 2.5 cm wide and placed in a windowless chromatogram scanner, model RSC-5B, Atomic Acess. Inc., for detection of radioactivity. Identically developed strips were dipped into 1 per cent alcoholic resorcinol: 0.2 N HCl (1:1 v/v) for detection of reducing sugars and sucrose (5).

For two dimensional chromatography, which was later used to obtain better separation and, hence, identification of labeled metabolites, 100 µl aliquots of the concentrated extract were used. Detection and location of labeled compounds on the two dimensionally developed chromatograms could not be made by a thin window survey meter because of the small amount of carbon-14 fixed and the low energy of its emitted

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1. PPO = 2,5-diphenyloxazole

   POPOP = 1,4-bis-2-(5-phenyloxazolyl)-benzene

beta particles. Autoradiography using no screen X-ray film would in all probability have given specific information regarding detection and location of the labeled metabolites, but it had the disadvantage of time. It was calculated that for the amount of carbon-14 fixed, extracted, and chromatographed, a period of 3 to 4 months would be required to obtain suitable autoradiographs.

A more rapid method was used utilizing a Baird-Atomic model 750 automatic sample changer and counter. This instrument has the additional advantage of a low background count rate of approximately 15 cpm at an operating voltage of 1500 volts. By cutting the two dimensionally developed chromatograms into numbered squares and counting them in the automatic counter, the areas of radioactivity could be identified. Therefore, 100 µl aliquots of the concentrated extracts were added by means of repeated applications onto one corner, 1.5 cm from each edge of the sheet of Whatman No. 1 chromatography paper (23 cm x 25 cm). In several instances the replicated spot was sprayed with a 1.0 per cent phosphatase solution to ascertain if any labeled metabolites could be freed from their phosphorus moities. After replication and spraying the chromatograms were formed into cylinders and developed two dimensionally by ascending chromatography. The first dimension solvent was water saturated phenol (100 gm phenol: 20 ml water w/v). When the aqueous phenol had ascended to 23 cm the chromatograms were removed from the solution and dried in a blower-ventilated oven at 72°C to remove the phenol. Upon drying, the chromatogram cylinders were opened, turned 90°, and reformed into cylinders. They were placed in the second dimension solvent of n-butanol: acetic acid: water (4:1:5 v/v/v) and removed when the solvent had ascended the full length of the chromatogram. The

chromatograms were then steamed for 10 minutes to remove the acetic "swamp" acid and air dried.

After steaming and drying, the chromatograms were cut into squares 3 cm on an edge, numbered, and run through the automatic sample counter. To calibrate count rates of the total fixed activity using the automatic sample counter, 100 µl of extract were evenly distributed over a solvent treated blank chromatogram square this same size and counted in the counter. The counter was pre-set at 300 counts and the time required for the activity of each square to attain this number was recorded. Dividing the pre-set count by the time required gave the activity in counts per minute. These counts, corrected for background, were recorded on a correspondingly numbered master card. In this manner an activity map of the chromatogram was obtained. Control chromatograms of known amino acids, tri- and dicarboxylic acids, and sugars were run with each extract chromatogram. Amino acids were detected by spraying with a 0.25 per cent solution of ninhydrin (1,2,3,-triketohydrindene) in n-butanol. The organic acids were detected by spraying with 0.1 per cent methyl red solution in ethanol adjusted to pH 8 with NaOH. The spray reagent for detecting phosphorus compounds consisted of 20 ml of 10 per cent ammonium molybdate added to 3 ml of conc. HCl followed by 5 gm of ammonium chloride (5).

From these controls and various reference sources (4, 7, 18), possible metabolites could be assigned to each square exhibiting radioactivity. The source of the activity was then eluted with water from the square and the radioactive elutant co-chromatographed with a known compound or compounds which were believed to be similar or identical with the unknown labeled metabolite. By such means the identity of the

unknown metabolites could be established. The area bearing the identified radioactive metabolite was cut from the chromatogram strip and its activity counted in the automatic sample counter. All count rate data reported are from eluted and co-chromatographed metabolites and, hence, are comparable. Several elutions and co-chromatographic analyses had to be made in some instances before identification could be established. The co-chromatographing was done one dimensionally with n-butanol: acetic acid: water (4:1:5 v/v/v).

RESULTS

Preliminary Observations

Preliminary studies indicated three general findings pertinent to the work reported herein. One, that no detectable $C^{14}O_2$ was found in the nutrient medium after it was subjected to extraction and lyophilization, two, that the $C^{14}O_2$ was completely liberated from its radioactive source, and three, at least a 10 fold increase in carbon-14 fixation was obtained in pollen grains which had germinated to produce pollen tubes. These observations were made under the experimental conditions maintained in this study.

Total Fixed Activity

It was originally thought that differential counts could be obtained between exposures made in the light and those made in the dark. The thesis here being that comparable studies have shown that the magnitude

of respiratory fixation is very small; the amount of C^{14} fixed in several hours in the dark being no greater than in a few seconds of photosynthesis at high light intensity at the same concentration of CO_2 (7). Consequently, any fixation above the respiratory fixation should give higher count rates. No consistent results, however, were obtained. Table 1 shows the total fixed activity of lupine pollen exposed to 11 μc of $C^{14}O_2$ for 90 minutes. All count data reported in this study are corrected for background.

Table 1. Total fixed activity of L. luteus pollen extracts from exposure to 11 μc of $C^{14}O_2$ for 90 minutes (cpm/100 μl).

Exposure Number	Exposure Condition	
	Light	Dark
1	3,045	1,979
2	2,851	2,505
3	1,825	1,888
4	2,000	1,758

These results clearly indicate that carbon-14 was being assimilated by the germinating pollen grains. It was determined, however, that these differential counts did not offer substantial evidence for greater fixation in light than in darkness in spite of the fact that these results showed greater fixation apparently occurred more often in the light. When the extracts from these 11 μc exposures were chromatographed and eluted, their fixed activity was found to be too low for the identification of their labeled metabolites.

In order for labeling of sufficient activity to be found after extraction, chromatography, and elution, the pollen was exposed to 20 μc

of $C^{14}O_2$ for 45 minutes. Comparable exposures at this activity were also made in an atmosphere of nitrogen. A nitrogenous atmosphere was used on the assumption that respiratory fixation would be markedly inhibited and, thus, enhance, relatively, a greater expression of any products of photosynthetic fixation as determined in later chromatographic analyses. Table 2 shows the total fixed activity of pollen exposed to 20 μc of $C^{14}O_2$ under aerobic and anaerobic conditions.

Table 2. Total fixed activity of *L. luteus* pollen extracts from exposures to 20 μc of $C^{14}O_2$ for 45 minutes under aerobic and anaerobic conditions (cpm/50 μl).

Exposure Number	Exposure Conditions			
	Light		Dark	
	N_2	Air	N_2	Air
1	962	3,673	1,814	6,724
2	1,679	3,089	1,893	6,006

The situation based on differential count data (Tables 1 and 2) under a normal atmosphere was confused but definitely did not suggest photosynthetic fixation of CO_2. A marked reduction of carbon-14 fixation occurred in a nitrogenous atmosphere (Table 2).

One Dimensional Chromatography

Although extracts from the 11 μc levels of exposure did not produce enough fixation for a critical study of those metabolites labeled, they provided pertinent information when subjected to one dimensional chromatography without subsequent elution and co-chromatographing.

Since sucrose was present in the nutrient medium and was non-volatile it could survive lyophilization and be co-chromatographed with those radioactive substances present in the extract. Sucrose is also a metabolite found labeled only in photosynthetic carbon-14 fixation. Consequently, it was considered advisable to determine if such free sugars as sucrose, glucose, and fructose were labeled. The latter two were included because of their chemical relation to sucrose and their similar reaction to the chromatographic detection reagent employed. Developed chromatogram strips from the 11 μc exposure extracts showed no radioactivity was associated with the free sugars when analyzed in a windowless chromatogram scanner. Fructose and glucose may have been present as metabolites or possibly arose by hydrolysis of sucrose by the acetic acid used as one of the solvents in the developing solution.

While no activity was found in the free sugar areas (lower central portion) of the chromatograms, radioactivity was found on the lower, upper central, and top portions of the developed strips. Activity in these areas indicated the presence of tricarboxylic acids in the top portion, sugar phosphates and phosphohydroxy carboxylic acids in the lower, and amino acids in all three portions. When the chromatogram strips were sprayed with ninhydrin, amino acids were shown to be present in all three portions of the radiograms. Spraying with other detecting reagents for organic acids and phosphorus compounds gave negative results which indicated their absence. A more plausible assumption is that, if present, they were there in such small quantities that these means of detection were not sufficiently sensitive to prove their presence. In any event, no labeled free sugars were detected. This constituted reasonable evidence against any photosynthetic fixation of $C^{14}O_2$

by the pollen of yellow lupine.

Two Dimensional Chromatography and Identification of Labeled Metabolites

The employment of higher activity levels (20 μc and 500 μc per exposure) combined with two dimensional chromatography and subsequent elution techniques provided the most informative results as to specific metabolites labeled. By these techniques, labeling was found in such organic acids as malic, succinic, fumaric, citric, and isocitric as well as in amino acids as alanine, aspartic acid, glutamic acid, proline, and serine.

Table 3 gives the activity of the labeled metabolites and their percentage of the total fixed activity from a 45 minute exposure to 20 μc of $C^{14}O_2$.

Table 3. Radioactivity of labeled metabolites and their percentage of total fixed activity from extracts of *L. luteus* pollen exposed to 20 μc of $C^{14}O_2$ for 45 minutes.

Labeled Metabolite	Exposure Conditions: Light — Total fixed activity 750 cpm/100 μl		Dark — Total fixed activity 1,450 cpm/100 μl	
	Cpm	% Total fixed activity	Cpm	% Total fixed activity
Malate	89	12.0	148	10.0
Succinate	39	5.0	39	3.0
Isocitrate and citrate	28	3.8	63	4.4
Fumarate	15	2.0	20	1.5
Alanine	5	0.8	43	3.0
Aspartate	27	3.6	30	2.1
Glutamate	88	11.7	151	10.4
Proline	50	6.7	113	7.8
Serine	17	2.3	120	8.3

Not all areas showing radioactivity on the chromatograms of the lupine pollen extracts gave count rates sufficient to survive elution and co-chromatographing for identification of labeled metabolites. These results provided no clarification of the type of CO_2 fixation resulting in their formation.

In many comparable studies, e.g. (4, 20), an effort was made using phosphatase to release any labeled metabolites from their phosphorus moities. Since triose and hexose phosphates arise by photosynthesis, it was deemed advisable to employ such a technique. Treatment of chromatograms with phosphatase, however, did not release additional labeled metabolites or alter the pattern of activity.

Comparable chromatograms of daylily (Hemercallis fulva Linn.) and petunia (Petunia hybrida Vilm) pollen exposed to 20 μc of $C^{14}O_2$ for 45 minutes presented the same general pattern of radioactivity as did comparable exposures of yellow lupine. The daylily and petunia pollen had not been induced to germinate, however. The location of the radioactivity indicated the carbon-14 had been fixed in malate, succinate, fumarate, aspartate, glutamate, alanine, and serine. The total radioactivity was much less, however, with these pollens than with lupine, being only 5 to 10 counts above background, and consequently, when these areas were eluted and co-chromatographed sufficient amounts of substances showing radioactivity could not be detected for identification of labeled compounds.

In those instances in which the lupine pollen was exposed in an atmosphere of nitrogen at 20 μc of $C^{14}O_2$, the developed chromatograms exhibited the same general pattern of activity as those exposed in a natural atmosphere. The radioactivity comprising this activity pattern

also was too low for positive identification of the labeled metabolites concerned.

Chromatograms of lupine pollen exposed to 500 μc of labeled CO_2 for a comparatively short interval (1.5 minutes) gave pertinent results. Table 4 shows the activity and percentage of fixed activity of metabolites from short exposures with $C^{14}O_2$ of high specific activity.

Table 4. Radioactivity of labeled metabolites and their percentage of total fixed activity from extracts of L. luteus pollen exposed to 500 μc of $C^{14}O_2$ for 1.5 minutes.

Labeled Metabolites	Exposure Conditions: Light — Total fixed activity 200 cpm/100 μl		Exposure Conditions: Dark — Total fixed activity 432 cpm/100 μl	
	Cpm	% Total fixed activity	Cpm	% Total fixed activity
Malate	57	28.5	113	27.5
Citrate	15	7.5	51	11.8
Aspartate	50	25.0	130	30.0
Glutamate	20	10.0	35	8.1

The high level of radioactivity found in malate and aspartate and the relative absence of numerous other labeled metabolites are in close agreement with findings of other investigators (6, 17) regarding those compounds first labeled in respiratory fixation using labeled carbon dioxide. In all cases, the results showed a markedly similar pattern of radioactivity of the chromatograms regardless of the conditions of exposure or species of pollen used. This activity pattern and the labeled metabolites composing this pattern constitute strong evidence for only a respiratory fixation of carbon dioxide occurring in the

pollen grains of yellow lupine.

DISCUSSION

Confirmatory Studies

A survey of the literature showed that no confirmatory investigations had been made. Ruhland and Wetzel used two methods of detection, luminescent microscopy and microchemical reactions which they believed conclusively proved the presence of chlorophyll in the generative cell of the lupine pollen grain. An attempt was made to repeat both tests.

A luminescent study was made with a Leitz-Wetzler ultraviolet microscope at the University of Kansas. Pollens from yellow lupine (Lupinus luteus Linn.), geranium (Pelargonium horitorium Bailey), and petunia (Petunia hybrida Vilm) were observed. In the pollen of lupine, where the cell contents were shown as a yellow-green matrix, the spindle-shaped generative cell was identified by its orange-red fluorescence which contrasted in some degree to the distinct reddish fluorescence reported by Ruhland and Wetzel. They also reported being able to observe the generative cell after it moved into the pollen tube, but this was not observed in the present study. The geranium pollen exhibited a uniform orange fluorescence while the petunia had a uniform yellow fluorescence, but in neither case were internal structures distinguishable.

The silver reduction-impregnation technique employed by Ruhland and Wetzel for chlorophyll identification was also used on pollen of the lupine. No positive results were obtained.

Since the two chlorophyll detection methods of the original study could not be duplicated, an alternative method was tried. This method involved paper chromatographic separation of chlorophyll (10). Using an extract of bean leaf as control, a distinct separation of the plastid pigments into chlorophyll a and b, carotene, and xanthophyll was obtained. Aliquots containing as little as 0.1 mg of bean leaf could be replicated and still contain sufficient pigment for identification.

The amount of chlorophyll in the lupine pollen is small, hence, pollen was collected from mature blossoms, quick frozen in a test tube held in a liquid air bath, and stored at -20°F until 100 mg of pollen was obtained. Attempts to demonstrate chlorophyll by paper chromatography were unsuccessful. To ascertain if the low temperature of and interval of storage were factors, 45 mg of freshly collected pollen was treated to the same chromatographic procedures. Again, the results were negative.

As a result of these confirmatory studies, no definite proof was obtained that the pollen of yellow lupine contains chlorophyll.

Carbon-14 Fixation Studies

It was soon shown that differential counts between the total fixed activities of light and dark exposures did not demonstrate the occurrence of photosynthesis in pollen grains reported to contain chlorophyll. Total activity studies proved, however, that carbon-14 was fixed in these grains.

The assumption that germinating pollen grains assimilate greater amounts of CO_2 than non-germinating ones was substantiated by a 10 fold

increase in those germinating as was indicated in preliminary aspects of this study. The process of germination is likely accompanied by an increased rate of respiration giving rise to a more rapid turn over of metabolites and possibly a greater production of metabolites through respiratory fixation.

Chromatographic techniques were more satisfactory in attacking the problem of photosynthetic activity in pollen of yellow lupine than was the use of total activity counts. The use of one dimensional chromatography did not demonstrate satisfactorily the absence of any photosynthetic activity, but did indicate that carbon fixation mechanism was more likely respiratory than photosynthetic.

The use of two dimensional chromatography and subsequent elution techniques for identification of the labeled metabolites produced results showing that carbon-14 fixation was likely through respiratory means only. As expected in respiratory carbon fixation, the labeled compounds involved intermediates of the Krebs cycle, aspartate (oxaloacetate), citrate, isocitrate, glutamate (α-ketoglutamate), succinate, fumarate, and malate. Labeled proline was also found, but it is not considered an intermediate of the Krebs cycle. The presence of the labeled proline was possibly to be expected because it has been reported quite abundant in some pollen (2, 3) and can arise from glutamate in several dehydration reactions. The appearance of such labeled metabolites and the absence of label among the triose phosphates, hexose phosphates, hexose diphosphates, sucrose, and other free sugars is rather formidable evidence against photosynthesis occurring in the pollen of yellow lupine.

It has been reported that the absence of respiratory intermediates

is quite striking when algae or barley leaves photosynthesize anaerobically in light (7). Anaerobic exposures of lupine pollen to labeled $C^{14}O_2$, in an atmosphere of nitrogen, were made in an attempt to enhance any possible photosynthetic activity by hindering the respiratory fixation. Resulting radioactivity maps based on the two-dimensional chromatograms, both from light and dark exposures to labeled carbon dioxide, were identical for exposures under both nitrogenous and natural atmospheres. The total fixation produced in a N_2 atmosphere was often 50 per cent less than that produced under a natural atmosphere. This similarity in the radioactivity pattern obtained between light and dark exposures to $C^{14}O_2$ and also under aerobic and anaerobic conditions further demonstrated that carbon-14 fixation in yellow lupine pollen is very likely by respiratory fixation.

It was found in 45 minute exposures of lupine pollen to 20 μc of $C^{14}O_2$ in both light and darkness that much of the recovered radioactivity was in malate and glutamate, each having approximately 11 per cent of the total activity. Proline, which is not a Krebs cycle intermediate, showed marked radioactivity and accounted for approximately 7 per cent of the total fixed activity. Likely the large amount of proline often found in pollen grains is related to this situation. Individual metabolites (organic and amino acids) showed comparable labeling, in specificity and degree, in both light and darkness although certain discrepancies were found. In general the comparable extent of labeling for a specific metabolite under both situations is a characteristic of respiratory fixation although in green plants in light the amount of metabolite fixed may be less.

It was found in the 1.5 minute exposure of lupine pollen to 500 μc

of $C^{14}O_2$ in both light and darkness that malate and aspartate contained approximately 55 per cent of the total radioactivity which was about equally divided between them. The remaining label was found primarily in glutamate and citrate. The fact that the label was almost exclusively found in these metabolites in both light and dark exposures represents additional confirmatory evidence that the pollen of lupine assimilates carbon dioxide only by respiratory fixation. If photosynthesis had occurred in any extent, the labeling in malate and aspartate should have occurred to a lesser degree and fixed carbon-14 would have been found in the triose and hexose phosphates and in sucrose. The marked degree of labeling in malate and in aspartate is in agreement with the findings of Saltman et al. (17) from their short-interval respiratory fixation studies on succulent plants.

The technique of locating the carbon-14 by making counts on small chromatogram squares in an automatic sample counter proved both rapid and satisfactory.

In the techniques used involving the elution and co-chromatographing of materials from chromatograms, or selected squares from same, there was always some loss of radioactivity. All count rates reported, however, are from eluted and co-chromatographed materials and, hence, comparable. This loss occasioned by the techniques employed may have important implications. Ruhland and Wetzel stated that the chloroplast in the pollen grain of yellow lupine was only 0.2 to 0.3 μ in diameter. The small amount of chlorophyll in these minute plastids would in likelihood synthesize only small amounts of metabolites, if any. Thus, the possibility exists of certain metabolites being present in such small amounts that they could not be properly evaluated by the methods

employed. If such metabolites were present and were properly identified, they would have constituted pertinent evidence of photosynthetic carbon fixation having occurred. A second possibility in accounting for the absence of photosynthetic products is that one or more enzyme systems have been lost by the pollen grain, thus inhibiting carbon reduction by photosynthesis. A third possibility is that the German workers did not observe chorophyll in the pollen grains of those species where they reported it to be present. Nevertheless, the results obtained by the methods employed constitute evidence strongly indicating that only a respiratory fixation of carbon dioxide occurs in the pollen of yellow lupine.

SUMMARY

Carbon-14 fixation studies were made with pollen of yellow lupine (Lupinus luteus) to ascertain whether the chlorophyll reported to be present in these grains was functional photosynthetically.

Luminescent microscopy, microchemical reactions, and pigment chromatography were used in attempts to confirm the presence of chlorophyll in the pollen of yellow lupine. Only luminescent microscopy gave indications of its presence and this cannot be considered as conclusive proof.

Total fixed radioactivity counts of the extracts from pollen of yellow lupine exposed to $C^{14}O_2$ in both light and darkness showed carbon-14 was fixed in these grains. Differential counts between extracts from light and dark exposures, however, did not present reliable evidence as to the occurrence of photosynthesis in lupine pollen.

The methods used disclosed a marked similarity of the areas of radioactivity on chromatograms from light and dark exposures to $C^{14}O_2$ under both aerobic and anaerobic conditions. The absence of labeled free sugars, triose phosphates, and hexose phosphates, and the specificity of labeling in certain intermediates of the Krebs cycle and their derived amino acids provide strong evidence for only a respiratory fixation of carbon dioxide in lupine pollen. These results, however, do not preclude the possibility that a more sensitive method of detection and evaluation could have demonstrated photosynthetic fixation in the lupine pollen.

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CARBON-14 FIXATION IN THE POLLEN OF YELLOW LUPINE (*LUPINUS LUTEUS* LINN.)

by

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The object of this investigation was to study carbon-14 fixation in the pollen grains of yellow lupine (Lupinus luteus) to determine if the chlorophyll, which it is reported to contain, is functional in photosynthesis.

Pollen was collected from greenhouse-grown plants, moistened with a sucrose medium, germinated on an etched glass plachet, and exposed to $C^{14}O_2$ both in light and darkness. Exposures of 90, 45, and 1.5 minutes were made at 11 μc, 20 μc, and 500 μc of $C^{14}O_2$ respectively. The 11 μc and 20 μc levels were obtained from $Na_2C^{14}O_3$ (1 to 5 mc/mM) and the 500 μc level from $BaC^{14}O_3$ (29 mc/mM) by acidification with lactic acid introduced through a syringe-septum arrangement in the chamber wall. After the prescribed exposure the chamber was opened, residual $C^{14}O_2$ swept away, and the pollen immediately extracted by dropping the planchet into boiling 80 per cent ethanol. After homogenization, the resulting extract was concentrated in a lyophilizer.

Total activity counts were made on extract aliquots in a liquid scintillation spectrometer or by evenly distributing the aliquot on squares (3 x 3 cm) of chromatogram paper and counting in an automatic sample counter.

Strips taken from a one-dimensional chromatogram made from 11 μc exposure extracts were analyzed in a chromatogram scanner for labeled free sugars. Two-dimensionally developed chromatograms of the 20 μc and 500 μc exposure extracts were cut into squares (3 x 3 cm) and their activities counted in the automatic sample counter. The counts were recorded on a correspondingly numbered master card. Thus, activity maps were obtained in lieu of autoradiograms. Identically chromatographed but known organic acids, amino acids, and sugars were used in

tentatively locating and identifying the unknown labeled metabolite(s). The tentatively identified labeled metabolite(s) was then eluted, concentrated, and co-chromatographed one-dimensionally with the comparable known compound(s). Upon definite identification the area of the metabolite was removed from the chromatogram and its activity counted in the automatic sample counter. All count data reported are from eluted and co-chromatographed metabolites and, hence, are comparable.

Luminescent microscopy, microchemical reactions, and pigment chromatography were used in attempts to confirm the presence of chlorophyll in the pollen of yellow lupine. Only luminescent microscopy gave indications of its presence and this cannot be considered as conclusive proof.

Differential counts of total fixed activity between light and dark exposures to $C^{14}O_2$ gave confusing results, but definitely did not indicate the occurrence of photosynthesis in lupine pollen.

Analyzing the one-dimensional chromatogram strips from 11 μc exposures showed no labeling of free sugars, but indicated labeling in certain amino acids, organic acids, and phosphorus compounds.

Exposing lupine pollen to 20 μc and 500 μc for 45 and 1.5 minutes, respectively, provided the qualitative evidence sought as to labeled metabolites. From the longer exposure it was determined that malate, citrate, isocitrate, succinate, fumarate, alanine, glutamate, aspartate, proline, and serine contained labeled carbon-14. With the shorter exposures only four metabolites (malate, aspartate, citrate, and glutamate) were labeled to any extent, the malate and aspartate showing marked labeling.

The absence of labeled free sugars and certain phosphates and the

specificity of labeling in certain intermediates of the Krebs cycle and their derived amino acids provided strong evidence for only respiratory fixation of carbon dioxide in lupine pollen.